Scholars Journal of Applied Medical Sciences (SJAMS) **ISSN 2320-6691 (Online)**
Sch. J. App. Med. Sci., 2017; 5(7A):2520-2526 **ISSN 2347-954X (Print)**

(An International Publisher for Academic and Scientific Resources)
www.saspublishers.com DOI: 10.36347/sjams.2017.v05i07.008

Original Research Article

Correlation of Indirect Immunofluorescence & Line Immunoassay Method in Detection of Autoimmune Diseases: an Observational Study at a Tertiary Care Teaching Hospital

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Abstract: Detection of antinuclear antibody (ANA) is one of the diagnostic criterias for autoimmune rheumatic diseases (ARD). Both indirect immunofluorescence (IIF) and line immunoassay (LIA)methods are useful for this purpose. However, there are lack of comprehensive data comparing these two methods in autoimmune diseases in Indian population. The aim of this study was to compare the ANA IIF patterns with LIA serum antibodies and to find a definite correlation between the these two methods.A total 662 serum samples of patients from a random east Indian population at a tertiary are institute suspected for rheumatic diseases were subjected for ANA testing by indirect immunofluorescence method and/or line immunoassay during the prospective cross sectional study period of 12 months. Out of 662 samples received, only 394 cases were analyzed for both ANA by IIF method and line immunoassay. Among these 138 (35.02%) were ANA-IIF positive and 114 (82.6%) were also line immunoassay positive. The homogenous pattern was the most common(n=60;52.6%) ANA pattern. The second most common was the speckled (*n* = 46; 40.3%) pattern. Different combinations of specific autoantibodies were observed in association with these ANA patterns. In our study, 24(17.3%) of the ANA-IIF positive samples showed negativity with line immunoassay. ANA-IIF negativity was observed in 256 of the total 394 samples under study. Of these, 38(14.8%) exhibited positivity with line immunoassay. 218 samples were negative for both ANA and line immunoassay. The fluorescence patterns of ANA IIF can predict the presence of certain specific antibodies in the sera detectable by LIA . These correlations are of relevance for the diagnosis of a specific rheumatic disease and help in avoiding costly investigative procedures unless needed.
Keywords: Antinuclear antibody test, Indirect immunofluorescence, Line immunoassay.

INTRODUCTION:

Spectrum of antibodies are produced by a patient with autoimmune diseases such as Systemic Lupus Erythematosus (SLE), scleroderma, CREST syndrome(Calcinosis, Raynaud's phenomenon, Esophageal motility abnormalities, Sclerodactyly and Telangiectasia), Sjogren's syndrome, Mixed connective tissue disease (MCTD), Polymyositis and Dermatomyositis[1].

Though An arrey of laboratory tests are available for detection of antinuclear antibody (ANA), The indirect immunofluorescence (IIF) test is currently considered to be the "gold standard" for detecting ANAs in clinical practice due to high degree of sensitivity and specificity[1]. IFA detects antibodies to different nuclear and cytoplasmic antigens. Five to six indirect immunofluorescence nuclear patterns are commonly reported by most laboratories[1,2]. It's a manual assay requiring experienced skilled personnel and a fluorescent microscope which may not be available in many laboratories. Therefore, an Line immunoassay (ANA-LIA) is considered a suitable alternative to ANA-IFA. The advantages of LIA testing include the speed and simplicity as well as more consistent results than immunofluorescence cell

substrates. Some LIAs approach immunofluorescence in their sensitivity and specificity for the identification of ANAs. No technical expertise is needed to interpret the LIA readings. Drawbacks of LIA testing include reduced antigen diversity leading to decreased sensitivity [3].

The present study was undertaken to compare the detection of ANA by immunofluorecence assay using HEp-2 cell substrate and Line immunoassay in patients with suspected autoimmune diseases & Compare both methods to find if a definite correlation exists between the two methods.

MATERIALS AND METHODS:

A total 662 serum samples of patients from a random east Indian population who sought medical help for rheumatic diseases as suspected by rheumatologists/ internal medicine specialists/dermatologists/ nephrologists or from any hospital department for a diagnosis of connective tissue diseases (CTD) were subjected for ANA testing by indirect immunofluorescence method and/or line immunoassay during the study period of 12 months from October 2016 to October 2017. The samples with a request for ANA by any method other than IIF, or samples received for single test or accompanied by a request with a non-rheumatic diagnosis were excluded from the study. Only samples received for both IIF and LIA testing are included in this study. This was a prospective cross sectional comparative study. The institutional ethical committee clearance was obtained prior to conducting the study.

Fresh fasting whole blood samples were collected with patient consent. Serum separated from the clottedblood samples by centrifugation was stored at 4°C if testing was planned within 72 hours or at -20°C for further testing after three days. ANA Immunoflurorescence assay was performed using theBio systems Immunofluorescence Kit. The procedure was carried out according to the kit manufacturer's instructions. The serum samples were diluted 1/40 times. 1 drop each of the control and test sera were placed on each slide wells, ensuring to cover it completely, so that anti nuclear antibodies in serum bind to the corresponding antigens present in the HEp-2 (human epithelial cell tumor line)cells coated on the slides. After an incubation of 30 minutes, the slide was drained and rinsed with phosphate buffered saline. Then a fluorescein labeled anti human globulin conjugate was added which helps in detection of antigen antibody complexes .After a further incubation for 30 minutes, the slide was rinsed, mounting medium was added and then it was examined under the fluorescent microscope. The different patterns of fluorescence observed were homogenous, speckled, nucleolar ,centromere and peripheral. The fluorescence intensity was scored semiquantitatively from 1+ to 3+ relative to the intensity of the positive control(3+) and negative control. Positive and negative controls were run with each test daily.The test result was discarded if the positive control sample failed to show the precise results[4].

ANA LIA was performed according to the Kit (Calbiotech) Manufacturer's instructions. Briefly, diluted (1:40)serum samples using HEp –2010 / liver biochip (Monkey) (EUROIMMUN AG)and conjugated with specific antihuman IgG (EUROIMMUNAG) present on Nylon strips coated with recombinant and purified antigens as discrete lines with plastic backing(EUROIMMUN AG) like nRNP / Sm, Sm, SSA,Ro-52, SSB, Scl-70, PM-Scl, PCNA, Jo-1, Centromere, dsDNA, nucleosomes, histones, ribosomal protein-P, anti-mitochondrial antibodies (AMA-M2) along with a control band. ANA specific antibody, if present, binds to the antigen. After all the unbound material is washed away and the enzyme conjugate is added to the antigen antibody complex. Then excess of enzyme conjugate washed off , the nylon strip was incubated to allow the hydrolysis of the substrate by the enzyme. The intensity of the colour generated is proportional to the amount of the IgG specific antibody in the sample[5].

RESULTS:

662 samples were received. Of these 662, only 394 cases were analyzed for both ANA by IIF method and line immunoassay in this study. Among these 394 samples, 138 (35.02%) were ANA-IIF positive in a 1:40 serum dilution. Of these positive ANA-IIF, 114 (82.6%) were also line immunoassay positive.

Total numberof samples with ANA IIF or LIA Positive=176
Number of ANA IIF positive with LIA positive=114
Number of ANA IIF positive with LIA negative=24
Number of ANA IIF negative with LIA positive=38
Number of ANA IIF negative with LIA negative=218

Table-1: various ANA patterns

ANA pattern	SAMPLES(%)
Homogenous	60(52.6%)
Speckled	46(40.3%)
Nucleolar	4(3.50%)
Centromere	3(2.63%)
Peripheral	1(0.87%)

The various ANA patterns seen in the positive samples with lineimmunoassay positivity are shown in Table 1.

The homogenous pattern was the most common ANA pattern, seen in 60 (52.6%)cases of the positive 114 samples [Figure1(a)]. The second most commonly occurring ANA pattern was the speckled (*n* = 46; 40.3%) pattern [Figure 1(b)]. In comparison with line immunoassay results of these samples , various combinations of specific auto-antigens were observed as depicted in figure 2 . Correlation with line immunoassay results of homogenous & speckled pattern showed combinations as shown in Table 2 &3. Specific combination of antigens was not observed in centromere and nucleolar patterns [Table 4]. ANA-IIF results exhibiting centromeric pattern were 3 (2.63%). 2/ 3 (33.6%) samples showed positivity for CENP-B (centromeric protein –B). RNP,Sm and SSA positivity was seen in the one case. Nucleolar pattern was observed in 4 (3.5%) samples[Table 4]. No specific combinations were found. Line immunoassay showed positivity for PCNA in 2 (1.75%), SSB , RNP and SSA in 1 sample (0.88 %). A single sample with rim /peripheral pattern showed positivity for SSA/Ro-52,dsDNA, nucleosomes and histones on line immunoassay. In our study, 24(17.3%) of the ANA-IIF positive samples showed negativity with line immunoassay. The ANA pattern observed in these cases were mostly homogenous 16 (66%), speckled pattern was seen in 6 (25%) cases and two (8.3%) cases exhibited nucleolar patter. ANA-IIF negativity was observed in 256 of the total 394 samples under study. Of these, 38(14.8%) exhibited positivity with line immunoassay. 218 samples were negative for both ANA and line immunoassay, though these samples were from patients who had rheumatic disease.

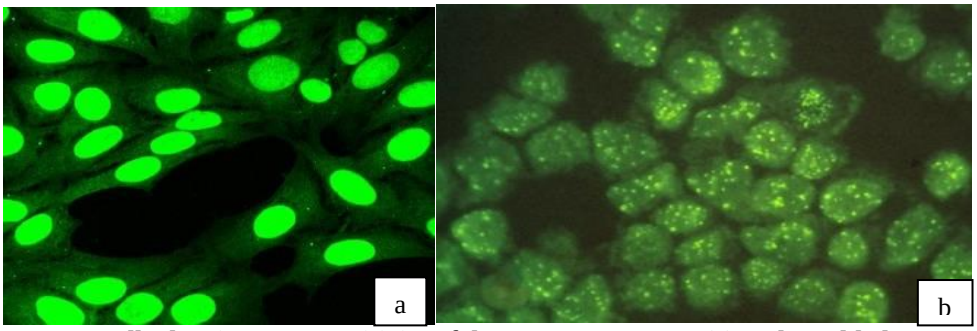

Fig 1: Hep-2 cells showing ANA positivity of homogenous pattern(a) and speckled pattern (b)

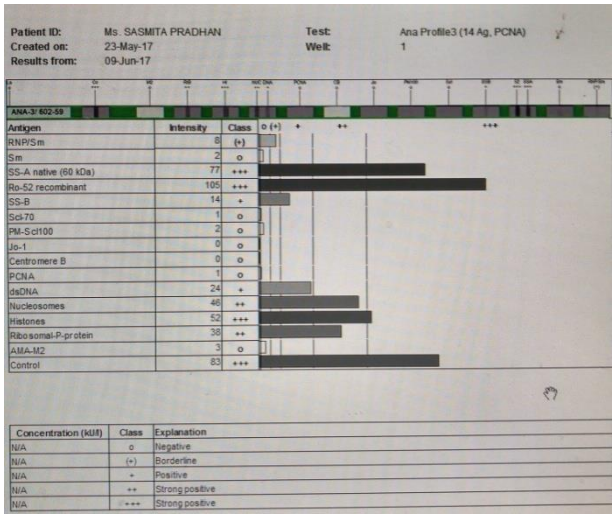

Fig-2: ANA profile strip showing anti bodies (2+/3+) against SS-A, Ro-52, SS-B , dsDNA, nucleosomes , histones and Ribosomal-P-protein read against control band (3+) in a sample showing a positive speckled pattern for ANA

Table-2:Immunoassay details of 60 samples with ANA-IIF homogenous pattern

Ds DNA, histone ,nucleosomes	17(28.3%)
Ds DNA, histone ,nucleosomes,SSA/Ro52	15(25%)
Ds DNA, histone ,nucleosomes,RIB	08(13.3%)
Ds DNA, histone ,nucleosomes,RNP/SM	04(6.6%)
RIB,SSA	16(26.6%)

Table-3: Immunoassay details of 46 samples with ANA-IIF speckled pattern

SSA/Ro52,SSB	21(45.6%)
RNP/Sm,SSA	14(30.4%)
SSA/RIB	07(15.21%)
RNP/Sm,SSA/Ro52,SSB	04(8.69%)

Table-4 : Immunoassay details of 8 samples with ANA-IIF centromere, nucleolar and rim patterns

Pattern	Sample no (percentage)	Line immunoassay specificity
Centromere	3(2.63%)	CENP-B (centromeric Protein –B) (2/3) RNP, Sm and SSA(1/3)
Nucleolar	4(3.50%)	PCNA (2/4) , SSB (1/4) RNP/ SSA (1/4)
Rim	1(0.87%)	SSA/Ro-52, dsDNA, Nucleosomes and Histones

DISCUSSION:

The presence of ANAs is a hallmark of rheumatic or autoimmune disease. Although some IIF patterns strongly suggest distinct specificities, additional tests are requested to demonstrate antibody reactivities against specific nuclear and cytoplasmic antigens to either support the diagnosis (disease specificity) or to identify subsets of patterns that are prone to particular disease manifestation (prognostic marker)[6].Detection of ANA by traditional assays are being replaced by newer technologies such as LIA, ANA-HEp-2. Because of its high sensitivity rates, a high false positive rate for ANAs is expected in IIF method(Rs 150 per test) and that makes interpretation of a positive test results difficult. LIA on the other hand though more specific method, its costly(Rs 1100 per test)[7]Perhaps the study that most closely resembles ours is by Slater and Shmerling, where ANA was performed on Hep-2 cell substrate at a titre of 1:40[8].In our study, 394of the 662 serum samples satisfied the definite selection criteria and processed for both ANA and line immunoassay tests.

There is a consistent female preponderance for autoimmune diseases. In this study also ANA positives were more among the females (82%) than the males (18%) . This correlates with the findings of Hayashi et al who reported that of the 111 patients with SLE,104 were women and were men, and the median age was 35 years[9]The likely explanation for this female preponderance is probably related to exogenous & endogenous hormonal changes[10].

In the present study, peak incidence of the ANA positives observed in 20 to 29 years age group of followed by 40 to 49 years. Priyadarshini et al. reported most of the ANA positives in the age group 21-30 years followed by 41-50 years[11].

114 (28.9%) of serum samples were both ANA and line immunoassay positive wherein the ANA pattern could be correlated with the presence of specific antibodies depicted by the immunostrip. Homogeneous, the most common ANA pattern observed in this study (*n* = 60, 52.6%)showed an association with dsDNA, nucleosomes and histones(*n* = 17) with variable intensities of SSA / Ro-52 (*n* = 15), RIB(*n* = 8) and RNP / Sm (*n* = 4). Thus, with a homogenous pattern, one can predict that the serum would have antibodies againstds DNA, nucleosomes and histones in 73% cases. The next common speckled pattern (*n* = 46, 40.3%) showed an association with SSA/Ro-52, SSB in all cases in varying combinations with RIB and RNP / Sm. Thus, one can predict the presence of SSA/Ro-52 and SSB in 100% of cases with speckled pattern. The centromeric and nucleolar ANA patterns were seen only in three(2.63%) & four(3.5%) cases each . Sixty six per cent of cases (*n* =2) with centromeric positivity showed centromeric protein-B. Thus one could predict the possibility of presence of CENP-B in 66% of centromere pattern cases. PCNA (*n*= 2, 1.75%), SSB (*n* = 1, 0.88%), RNP, SSA (*n*= 1, 0.88%) was seen with nucleolar pattern. As the numbers are very small, no definite correlation could be drawn between the IF pattern and the antibodies present in case of rim pattern.

Positive ANA-IIF with negative line immunoassay was interestingly noted in 17.3% of cases (*n* = 24). A positive result with ANA-IIF, together with negative results in line immunoassay, was also noted earlier and attributed to the fact that though by line assay we could differentiate ANA, it may sometimes miss the detection of rare ANAs[12]. Vos *et al*. have found3/32 ANA positive samples negative with line immunoassay but found positive for anti-dsDNA antibodies by FARR assay. Therefore, in the 24 cases studied here, the serum has probably, some antibodies other than the most commonly encountered 14 antigens. Review of the ANA patterns of these24 cases had shown that homogenous pattern was in 16, speckled in six and nucleolar in two. However, they need to be followed up to understand the significance before one attributes to ANA as a too sensitive test in comparison to line immunoassay .On the contrary, 38 sera positive for line immunoassay were negative by ANA-IIF. 29 of these sera showed SSA/Ro-52positivity while seven showed positivity for Scl-70. A similar observation was noted by Vos *et al*. and Hoffman *et al*[6,13].This is explained by the fact that line immunoassay is more sensitive for the detection of SSA/Ro-52 than ANA-IIF even when Hep-2000 cells are used. Screening with ANA-IIF also missed Scl-70 antibodies in a patient, which is of relevance in polymyositis. Scl-70 reactivity goes undetected or unreported with ANA-IIF as these antibodies give a cytoplasmic positivity rather than nuclear staining pattern on IIF and that ANA could have been reported as negative. This drives home a message that cytoplasmic staining pattern identification is equally important even though ANA is reported “negative” to pick upScl-70 antibodies[7].These imply that a certain degree of expertise is essential for the reporting pathologists while consistently reporting ANA.A protocol could be made where at least two pathologists should report independently and a consensus is taken before the report is signed out. Careful reporting of ANA is also advised to identify rare but specific ANA patterns . It may also be important to run the sera for line immunoassay if ANA is negative, but rheumatic disease is strongly suspected as is seen in 38 cases, which were ANA-negative but line immunoassay-positive[2,3]. LIA for ANA was positive in 152(38.57%) & IFA was positive in 138 (35.02%) patients suspected to have autoimmune diseases. LIA showed more positives than IFA the in the test group. The findings obtained in this study are comparable with those in western literature. There is fair degree of correlation between homogenous, speckled, nucleolar and centromere patterns and their corresponding antibodies in sera. A singlecase of rim pattern was insufficient to compare with line immunoassay in our study.

In the current study, comparing LIA with the gold standard IFA, the sensitivity of LIA was found to be 89% and specificity was 42%. Similar to the present study, Susan Copple et al showed that the Bio-Rad, Phadia, Aesku, and Inova ANA ELISAs demonstrated excellent screening sensitivities of 96.6%,96.6%, 90%, and 96.6%, respectively and specificities ranging from 36% to 94%[14].Another study by Jaskowski et al found that the sensitivity and specificity of different

ELISA kits ranged from 69.5% to 97.7% and81.4% to 97.9% respectively[15]. The lack of agreement between test methods may reflect differences in the array of antigens present in the various assays. An explanation for low specificity in our study may be due to the use of imported kits which gave a high cut-off concentration levels to differentiate a positive and a negative sample or it may be due to the limited number of antigens used in the LIA which may not reflect the autoantibody patterns of the local population or due to less sample size in our study. There may also be racial and/or ethnic differences in the autoantibody patterns found in different population groups[16]. The sensitivity of IFA was 91%.This higher sensitivity in IIF is usally due to use of HEp-2 cells which contain high concentration of nuclear and cytoplasmic antigens which results in. Thus it is understandable that LIA with its limited content of antigens may fail to detect certain antibodies which can be detected by IIF[18].

In our study anti dsDNA was the most common ANA detected (69%) and ANA against SS-A antigen being the second (34.2%). The dsDNA and Sm antigens are targets for autoantibodies in SLE, and considered to be highly specific for this condition[18].Isolated presence of Sm band in the absence of other SLE specific bands like anti-dsDNA may not be significant. The SS-A & nRNP band, which are found in a variety ofARDs, appear in only 20%-30% & 30%-40% of SLE patients . So it seems correlation with other band patterns is equally important in the interpretation of bands like SS-A & nRNP[19].In our study line assay showed positivity for antibodies in different combinations which implies that this method has the advantage to detect patients with overlap syndrome, giving more information about the ANA present in the patient sample than IIF, which needs greater expertise to detect different patterns of ANA in a single sample.

CONCLUSIONS:

ANA by IIF is the most cost - effective test for investigating rheumatic disease which can be used for screening purposes for patients in daily clinical practice. Its fluorescent pattern could also predict the presence of certain specific antibodies in the sera. These correlations are helpful not only in diagnosis of a specific rheumatic diseases and also keep lineimmunoassay as a secondary investigation especially for those patients who need them for prognostic significance.

Conflict of interest:

The authors declare no conflict of interest.

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